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Original article (Orijinal araştırma)

Distribution of entomopathogenic fungi inducing natural infections in *Orosanga japonica* (Melichar, 1898) (Hemiptera: Ricaniidae) in the Eastern Black Sea Region of Türkiye

Türkiye'nin Doğu Karadeniz Bölgesi'nde *Orosanga japonica* (Melichar, 1898) (Hemiptera: Ricaniidae)'da doğal enfeksiyona neden olan entomopatojenik fungusların dağılımı

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Abstract

Orosanga japonica (Melichar, 1898) (Hemiptera: Ricaniidae) is an important pest in the Black Sea Region in Türkiye. Despite the use of various methods and microbial factors from different sources to control this pest until recently, several pathogens have been detected. Entomopathogenic fungi are important microbial agents in pest control. In the present study, we isolated eighteen fungal strains from nymphs and adults of *O. japonica* with fungal infection collected in Rize, Trabzon and Artvin provinces of Türkiye in 2021-2022. According to morphological and molecular characterization based on internal transcribed spacer (ITS) sequences, all strains were identified as *Beauveria bassiana* (Bals.-Criv.) Vuill. (Ascomycotina: Hypocreales) (OJ1-OJ18). A screening test at 1×10^7 conidia/ml revealed that all the strains caused 75–100% mortality in nymphs and adults after 7 days. Further experiments were performed with the three most effective strains, all belonging to *B. bassiana* (OJ3, OJ7 and OJ15). The dose–response tests of the three fungal strains were carried out on *O. japonica* nymphs and adults at concentrations of $1 \times 10^{4-8}$ conidia/ml. As a result of the concentration-response, the *B. bassiana* OJ3, OJ7 and OJ15 strains presented mortality rates of 100, 98.84 and 100%, respectively, at the highest dose (10^9 conidia/ml) on the 10th day, and the calculated LC_{50} values of OJ3, OJ7 and OJ15 were determined to be 1×10^4, 1.4×10^4 and 0.3×10^3 conidia/ml against nymphs and 1.6×10^4, 1.4×10^4 and 0.4×10^3 conidia/ml against adults, respectively. The presence of *B. bassiana* detected natural infections and the efficacy trials conducted in the laboratory revealed that *B. bassiana* has potential for use against *O. japonica*.

Keywords: *Beauveria bassiana*, biological control, entomopathogenic fungi, *Orosanga japonica*

Öz

Orosanga japonica (Melichar, 1898) (Hemiptera: Ricaniidae), Türkiye'nin Karadeniz Bölgesi'nde önemli bir zararlıdır. Yakın zamana kadar bu zararlının mücadelesinde çeşitli yöntemler ve farklı kaynaklardan elde edilen mikrobiyal etmenler kullanılmasına rağmen, birkaç patojen tespit edilmiştir. Entomopatojenik funguslar, zararlı mücadelesinde önemli mikrobiyal etmenlerdir. Bu çalışmada, 2021-2022 yıllarında Türkiye'nin Rize, Trabzon ve Artvin illerinden mantar enfeksiyonu taşıyan *O. japonica* nimf ve erginlerinden on sekiz fungus izolasyonu yapılmıştır. Morfolojik ve iç transkripsiyon aralayıcı (ITS) dizilerine dayanan moleküler karakterizasyon sonucunda, tüm suşların *Beauveria bassiana* (Bals.-Criv.) Vuill. (Ascomycotina: Hypocreales) (OJ1-OJ18) olduğu tanımlanmıştır. Tarama testlerinde, 1×10^7 konidi/ml konsantrasyonunda tüm izolatların 7 gün sonunda nimf ve erginlerde %75-100 oranında öldürücülük sağladığı gözlemlenmiştir. En etkili üç izolat olan OJ3, OJ7 ve OJ15 ile gerçekleştirilen konsantrasyon-cevap testlerinde, *O. japonica* nimf ve erginleri üzerinde 1×10^4-10^9 konidi/ml konsantrasyon aralığında uygulamalar yapılmıştır. Konsantrasyon-cevap testleri sonucunda, 10. günde en yüksek dozda (10^9 konidi/ml) OJ3, OJ7 ve OJ15 izolatları sırasıyla %100, %98.84 ve %100 oranında öldürücülük göstermiştir. OJ3, OJ7 ve OJ15 izolatlarının LC_{50} değerleri nimfler için sırasıyla 1×10^4, 1.4×10^4 ve 0.3×10^3 konidi/ml; erginler için ise 1.6×10^4, 1.4×10^4 ve 0.4×10^3 konidi/ml olarak belirlenmiştir. Bu çalışmada, *B. bassiana* doğal enfeksiyonlarının tespit edilmesi ve laboratuvarda gerçekleştirilen etkinlik denemeleri, *B. bassiana*'nın *O. japonica*'ya karşı kullanım potansiyeline sahip olduğunu ortaya koymuştur.

Anahtar sözcükler: *Beauveria bassiana*, biyolojik mücadele, entomopatojenik fungus, *Orosanga japonica*

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Introduction

Orosanga japonica (Melichar, 1898) (Hemiptera: Ricaniidae), commonly known as invasive polyphagous, has emerged as a significant pest in Türkiye (Demir, 2009; Arslangündoğdu & Hizal, 2019). This invasive insect species, native to East Asia, has found its way to Turkish shores, causing concern among agricultural experts and farmers alike. Nymphs and adults are intensively found on many plant species including vegetables, fruits, and ornamental plants (Gjonov, 2011; Göktürk, 2018; Karataş et al., 2020). Its rapid reproduction rate and voracious feeding habits make it particularly destructive, leading to significant economic losses in agricultural areas. The pest, which was first detected in our country in 2006, reached a very significant population density in the next few years and spread throughout the Black Sea Region (Göktürk & Aksu, 2014; Ak et al., 2015; Göktürk & Mıhlı, 2015).

Entomopathogenic fungi are part of the fungal phylum Ascomycota and the order Hypocreales, which includes several notable genera such as *Beauveria* spp. and *Metarhizium* spp. These fungi have evolved specialized mechanisms to invade and colonize their insect hosts, leading to host mortality through a combination of direct damage and the production of potent toxins. They stand out as an environmentally friendly option instead of pesticides. The increasing application of integrated pest management (IPM) is motivated by its specificity, low toxicity to non-target beneficial organisms, and minimal environmental impact. Furthermore, their ability to persist in the environment and adapt to different ecological niches makes them attractive candidates for long-term pest control.

Numerous studies have been conducted in Türkiye on the identification, biology, distribution, and management of this pest (Demir, 2009, 2018; Güçlü et al., 2010; Ak et al., 2013, 2014; Göktürk & Mıhlı, 2015, 2016; Alev & Sezen, 2016; Göktürk, 2018, 2019, 2020; Göktürk et al., 2018; Akıner et al., 2019, 2020, 2021, 2022; Altaş & Ak, 2019; Arslangündoğdu & Hizal, 2019; Güney et al., 2020; Erper et al., 2022). Microbial natural enemies have significant effects on controlling pest populations in nature (Biryol et al., 2021; Sevim et al., 2012; Sönmez et al., 2016; Gençer et al., 2023). In support of this, several studies have demonstrated that entomopathogenic fungi exert highly lethal effects on their host organisms from which they are isolated (Yücel et al., 2018; Biryol et al., 2020, 2021). Despite these findings, research on the distribution of *O. japonica* cadavers infected with fungi in the Eastern Black Sea Region, the isolation of fungal strains from those cadavers, and investigations into the potential use of these fungi for biological control of the pest is extremely rare. In this study, we isolated eighteen fungal strains from *O. japonica* nymphs and adults with fungal infections provided from Rize (Ardeşen, Pazar, and İyidere), Trabzon (Of), and Artvin (Hopa) provinces in Türkiye, characterized them, and analyzed the pathogenicity of strains isolated from dead *O. japonica* collected from various fields in Türkiye.

Materials and Methods

Collection of insect samples

Orosanga japonica nymph and adult cadavers were collected from Rize (Ardeşen, Pazar and İyidere), Trabzon (Of) and Artvin (Hopa) in Türkiye, where insects are very densely transferred to the laboratory in sterile plastic tubes and stored at 4ºC until use. Individuals infected with mycosis from *O. japonica* nymphs and adults were used for isolating entomopathogenic fungi.

Healthy *O. japonica* nymphs were collected from tea plantation fields in Rize (Pazar) Province and transported to the laboratory in plastic containers with proper ventilation and nutrient content. They were then fed fresh tea leaves and shoots under laboratory conditions until the insecticidal activity tests were conducted.

Isolation and purification of fungal isolates

Fungal strains were obtained from mycosed *O. japonica* nymphs and adults using an inoculation loop and cultured on PDAY (potato dextrose agar + 1% yeast extract; Merck, Darmstadt, Germany). To suppress bacterial contamination, chloramphenicol was added to the medium at a concentration of 40 µg/ml. The inoculated Petri dishes were kept waiting at 28ºC until fungal colonies developed. Pure isolates were obtained by selecting morphologically distinct single colonies based on their infection patterns (Yücel et al., 2018). A total of eighteen isolates were purified and labeled with local codes (e.g., OJ1–OJ18). Stock spore suspensions were prepared from purified cultures and kept at - 80ºC.

Morphological characterization

The purified isolates were first assessed for colony morphology on Sabouraud-CAF agar (Liofilchem s.r.l., Italy). Microscopic features were examined using the slide culture method, which involved placing small fragments of the medium inoculated with fungal spores on glass slides and covering them with coverslips (Senthilkumar, et al., 2021). Following 4-5 days of incubation, fungal structures were directly observed under a light microscope. Additional morphological details, including conidial structures, were examined under phase-contrast microscopy after staining with lactophenol cotton blue. Identification was performed following the diagnostic guidelines of Humber (1997).

Molecular characterization

To genetically identify the isolates, the internal transcribed spacer (ITS) regions-comprising ITS1, 5.8S, and ITS2 were amplified. Spores from pure cultures were cultivated on Sabouraud CAF agar at 28ºC for seven days. Mycelial biomass was collected using sterile spatulas and transferred to microtubes. Genomic DNA was extracted from ~50 mg of fungal material using the ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research), following the manufacturer's instructions.

PCR sample was conducted using the primers ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), as detailed by White et al., (1990). Each 50 µl PCR reaction included genomic DNA, reaction buffer, $MgCl_2$, dNTPs, Taq polymerase, and primers. Amplification was performed using the thermal cycling protocol recommended for the kit. The reaction conditions were after a denaturation step at 98ºC for 30 s, 35 cycles of denaturation at 98ºC for 30 s, annealing at 50ºC for 30 s, extension at 72ºC for 1 min and a final extension at 72ºC for 10 min. PCR products were separated using 1.0% agarose gels containing ethidium bromide and visualized under UV illumination.

Following purification, Sanger sequencing was performed by ETKA Biotechnology (Samsun, Türkiye). Resulting sequences were analyzed for similarity using BLAST in the GenBank database (Benson et al., 2012). Multiple sequence alignments were performed using ClustalW2 in BIOEDIT v.7.2.5 (Hall, 1999). Phylogenetic relationships were inferred with the Maximum Likelihood (ML) method in MEGA v.11.0 (Tamura et al., 2013), and the results were compared to related taxa. The ITS sequences of all isolates were submitted to the NCBI database and assigned unique accession numbers.

Screening tests

Eighteen fungal strains isolated from *O. japonica* nymphs and adult cadavers were tested for efficacy against *O. japonica* nymphs and adults. Fungal samples were incubated on agar plate at 25ºC for 3 weeks. Fungal conidia were collected using a 0.1% Tween 80 (AppliChem) solution and subsequently counted under a microscope to determine concentrations. Fungal suspension was applied using a sprayer at 1×10^7 conidia/ml concentration. Thirty individuals were used for the whole treatment, and tests were conducted in triplicate at different times. Only 0.1% Tween 80 was utilized for the control tests. Mortality was monitored for 7 days following treatment.

Concentration-response tests

The isolates OJ3, OJ7, and OJ15, which showed the highest insecticidal activity in the screening trials, were assessed in concentration-response assays. In this study, conidial suspensions from fungal strains were diluted in a 0.1% Tween 80 solution to obtain five different concentrations ranging from 10^8 to 10^4 conidia/ml. These concentrations were applied to third-instar nymphs and adults of *O. japonica*. The experiments were conducted following the methodology outlined in the screening tests described above (Biryol et al., 2021).

Statistical analysis

Mortality rates were adjusted using the formula developed by Abbott (1925). The study data were examined using a one-way analysis of variance (ANOVA) and a post-hoc comparison with the least significant difference (LSD) test to identify differences between the means. Probit regression analysis was performed on the concentration–mortality data to estimate the median lethal concentration (LC50) (Finney, 1971). Statistical analyses were conducted with SPSS software version 25.0 for all experiments (IBM Corp., Armonk, NY, USA).

Results

A total of 18 fungal isolates were obtained from 263 (6.84%) *O. japonica* infected individuals (Table 1). These isolates were considered infected when mycelial growth extended beyond the cadavers. These isolates were obtained from *O. japonica* nymphs and adults naturally infected by fungi and collected from areas exposed to insect infestation (Figure 1). Morphological observations revealed that all fungal isolates exhibited characteristics consistent with *Beauveria* species. Identification was based on the morphological characteristics, specifically the appearance and size of spores grown on agar medium, following the criteria outlined by Humber (2012). The colonies appeared round, characterized by a white, powdery surface with subtly downy, concentric rings. Phase-contrast microscopy images of the fungal isolates and slide cultures are presented in Figure 2. The spores were globose to subglobose in structure.

Table 1. Information on sampling locations and individual counts of adults and nymphs

Location	Nymph individual number	Adult individual number	Fungal isolates
Rize (Ardeşen)	12	19	2 (OJ6 and OJ7)
Rize (Pazar)	17	26	5 (OJ1-OJ5)
Rize (İyidere)	37	41	3 (OJ9-11)
Trabzon (Of)	48	52	7 (OJ12-18)
Artvin (Hopa)	4	7	1 (OJ8)

Figure 1. *Orosanga japonica* adult with natural *Beauveria bassiana* infection.

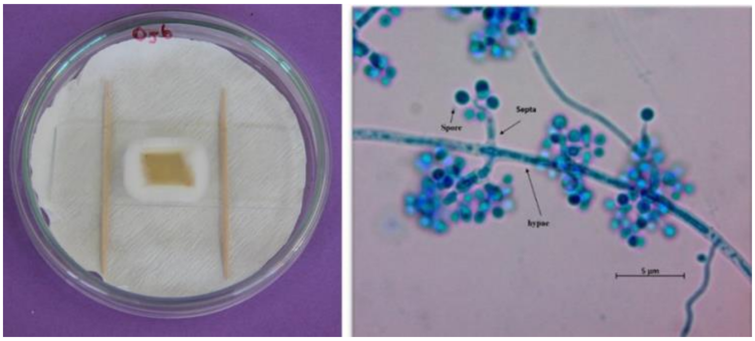

Figure 2. *Beauveria bassiana* morphological identification using the Bloc culture method (left) and light microscope image stained with lactophenol blue (right).

PCR amplification targeted the ITS1-5.8S-ITS2 gene region, and total DNA was extracted from all fungal isolates. The DNA fragments, approximately 530 base pairs in length, were successfully amplified and subsequently visualized using gel electrophoresis. The ITS sequences of the isolates were >99% similar to *B. bassiana* as a result of BLAST. In the phylogenetic analysis, all isolates were observed to be closely related to *B. bassiana* (Bals.-Criv.) Vuill. (Ascomycotina: Hypocreales) (Figure 3). The sequences were submitted to GenBank with accession numbers OR835539–OR835556.

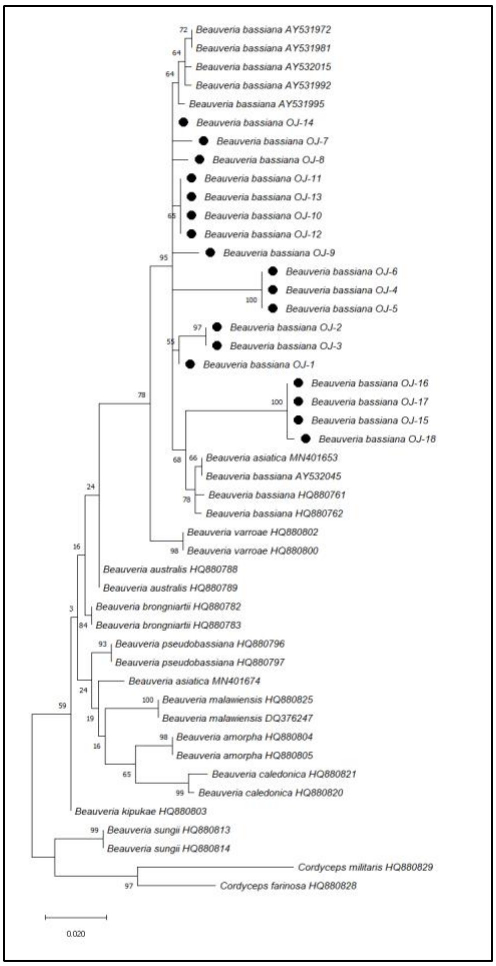

Figure 3. Phylogenetic relationships among *Beauveria bassiana* isolates and related taxa were inferred via Maximum Likelihood analysis using ITS1-5.8S-ITS2 sequences. Bootstrap values (1,000 replicates) are shown at nodes, and individual isolates are marked with black dots.

In a screening test conducted with 1×10⁷ conidia/mL, all strains resulted in mortality rates between 75% and 100% in both nymphs and adults after 10 days. Furthermore, experiments were carried out with the three most effective strains, all of which were *B. bassiana* species (OJ3, OJ7, and OJ15) (Figure 4). These strains were selected based on their spread on petri dishes and cadavers.

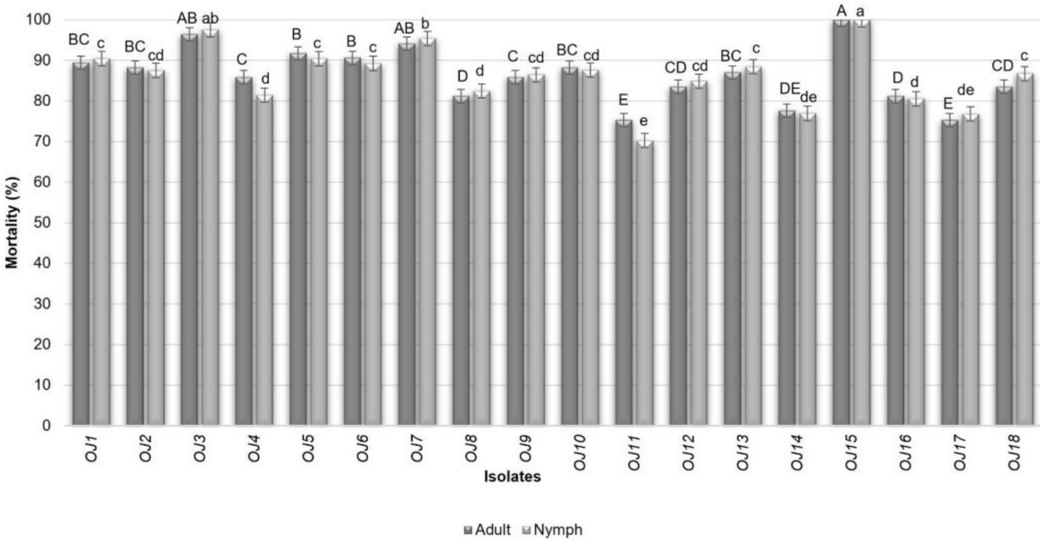

Figure 4. Insecticidal activities of *Beauveria bassiana* OJ1-18 isolates at 1 × 10^7 conidia/ml on nymphs and adults of *Orosanga japonica* within 10 days. Capital letters represent data for nymphs; lower case letters represent data for adults. Bars show standard error.

Eighteen strains of entomopathogenic fungi exhibited pathogenicity against *O. japonica* within seven days of inoculation, with mortality rates ranging from 75% to 100%. While all strains demonstrated varying mycosis rates, strains OJ3, OJ7, and OJ15 recorded the highest mortality rates (>94%, $p < 0.05$; Figure 4). However, these values were not significantly different from those of several other strains. The mortality rates of all tested strains significantly exceeded those of the control group within seven days ($F = 79.4$; df = 17, 36; $p < 0.05$). *B. bassiana* OJ3, OJ7, and OJ15 (the most virulent strains), resulted in mortality rates of 97.52%, 95.42%, and 100% in nymphs, and 96.47%, 94.12%, and 100% in adults, respectively.

In the dose-response assessment, these strains exhibited mortality rates of 100%, 98.84%, and 100%, respectively, at the highest concentration (10^9 conidia/ml) by the 10th day (Figure 5). The calculated LC50 values for OJ3, OJ7, and OJ15 were 0.3 × 10^5, 0.2 × 10^5, and 0.2 × 10^4 conidia/ml, respectively (Table 1).

Table 1. Concentration mortality rates of *Beauveria bassiana* strains on nymphs and adults of *Orosanga japonica*

Isolate	LC_{50} (conidia $ml^{-1)}$ (FL 95%)	Intercept	(*Slope* ± *SE*)	X^2	*df*	*n*
			Nymph			
OJ3	1×10^4 (0.06-15.3 ×10^4)	3.492	0.37±0.60	0.992	3	90
OJ7	1.4×10^4 (0.11-18.3 ×10^4)	3.291	0.41±0.56	0.995	3	90
OJ15	0.3×10^3 (0.0009-11.5 ×10^3)	4.132	0.33±0.78	0.996	3	90
			Adult			
OJ3	1.6×10^4 (0.12-21.5 ×10^4)	3.334	0.39±0.56	0.990	3	90
OJ7	1.4×10^4 (0.11-17.6 ×10^4)	3.274	0.41±0.55	0.994	3	90
OJ15	0.4×10^3 (0.001-14.8 ×10^3)	4.082	0.33±0.76	0.998	3	90

Abbreviations: *df*: degrees of freedom; *FL*: fiducial limit; *SE*: standard error; X^2: chi-square; *n*: Number of individuals.

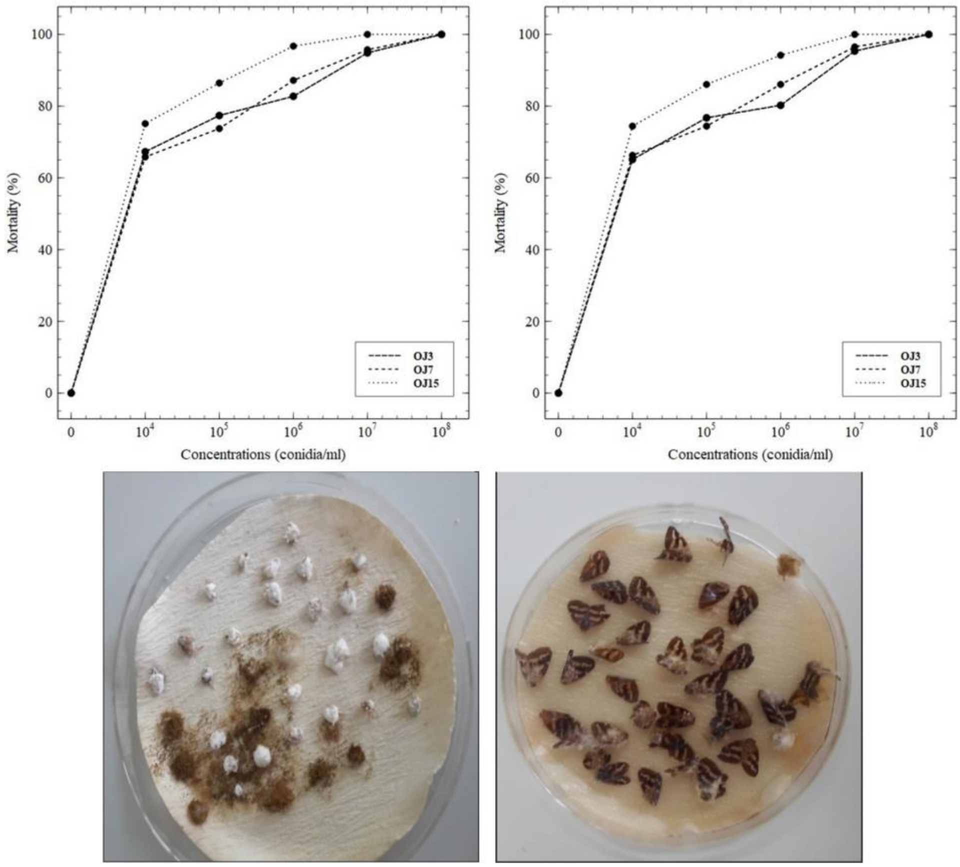

Figure 5. The insecticidal effects of the newly isolated *Beauveria bassiana* strains OJ3, OJ7, and OJ15 on *Orosanga japonica* nymphs and adults over a 10-day period. The fungal conidia concentrations tested ranged from 1 × 10^4 to 1 × 10^8 conidia/mL (above). Images of *Orosanga japonica* nymphs (bottom left) and adults (bottom right) exhibiting mycosis following infection with the *Beauveria bassiana* isolates in the bioassay.

Discussion

In recent years, a consistent increase in the population of the invasive species *O. japonica* was observed in the Eastern Black Sea region, leading to significant damage to various wild and cultivated plant species (Ak et al., 2015). The climate in the region where fungal infection samples are found is characterized by heavy rainfall, low temperature, and high humidity. These climatic features are highly conducive to the improvement of entomopathogenic fungi (EPFs) that can infect insects such as *B. bassiana* (Akıner et al., 2020). EPF conidia require moisture for germination and sporulation, with certain species specifically dependent on elevated humidity levels to initiate infection. Furthermore, rainfall plays a critical role in the dissemination of EPFs across insect populations (Goettel et al., 2005).

Beauveria bassiana, an entomopathogenic fungus, has shown considerable promise as a biological control agent for various insect pests. The pathogenicity of this fungus, when isolated from insect pests, varies between different strains, which affects its efficacy in pest control. Ribosomal RNA genes exhibit varying degrees of conservation, with spacer regions showing considerable divergence. The internal transcribed spacer (ITS) sequence is widely used to classify and identify fungi (Driver et al., 2000; Cai et al., 2013). Sevim et al., (2010) indicated that 23.3% of *B. bassiana* isolates from the eastern Black Sea region of Türkiye were obtained from various soil sources, and their identity was confirmed by analyzing the ITS1-5.8S-ITS2 region. Eighteen distinct *B. bassiana* isolates were identified in the current study, differing from those previously found in the eastern Black Sea region. These findings suggest that the region possesses a diverse range of entomopathogenic fungal strains, likely attributed to the high humidity levels.

In the present study, 18 entomopathogenic fungi were isolated. *B. bassiana* was found to be effective against a wide range of orders and pest species (citation). The results of studies on *O. japonica* indicate that various native isolates of *B. bassiana* exhibit high virulence against *O. japonica*, with laboratory tests revealing lethal effects at concentrations as low as 1 × 10^6 conidia/ml, resulting in 100% mortality within five days (Akıner et al., 2020; Erper et al., 2022). Akıner et al. (2020) reported that the leaf dipping method at the concentration of 1×10^6 conidia\ml yielded the most effective results, with LT_{50} values decreasing to 2.56 days for isolate 2. Similarly, for isolate 1, the LT_{50} values ranged from 6.634 days at 1×10^4 conidia/ml to 2.92 days at 1×10^6 conidia/ml, indicating a dose-dependent increase in virulence. In contrast, the dose-response assessment presented in the current study demonstrates a pronounced efficacy at higher concentrations, with mortality rates reaching 100%, 98.84%, and 100% at 1×10^9 conidia/ml for isolates OJ3, OJ7, and OJ15, respectively, by the 10th day. The calculated LC_{50} values for OJ3, OJ7, and OJ15 were significantly lower, ranging from 0.3×10^5, 0.2×10^5, and 0.2×10^4 conidia/ml, indicating higher virulence at lower concentrations when compared to Akıner et al. (2020). The discrepancies between the two sets of findings may be attributed to variations in experimental methodologies, isolate virulence, and application techniques.

Akıner et al. (2020) reported that the optimal concentration for the potential use of *B. bassiana* isolates as a biological control agent ranged from 1 × 10^6 to 1 × 10^8 conidia/ml, which aligns with the findings of the current study. Göktürk et al., (2018) observed that the biopesticidal efficacy of *B. bassiana* against nymphs and adults of *O. japonica* was relatively low, with mortality rates below 20%. In contrast, the 18 *B. bassiana* strains isolated in this study demonstrated a high level of efficacy against both nymphs and adults.

In this study, the fungal strains OJ3, OJ7, and OJ15 caused mortality rates of 100%, 98.84%, and 100%, respectively, when applied at the highest concentration of 1 × 10^9 conidia/ml by day 10. The corresponding LC_{50} values were estimated as 0.3 × 10^5, 0.2 × 10^5, and 0.2 × 10^4 conidia/ml for OJ3, OJ7, and OJ15, respectively. Gençer & Bayramoğlu (2022) similarly reported high insecticidal activity of *B. bassiana* isolates obtained to *Galleria mellonella* L. larvae collected from beehives, with mortality rates of 96.54% and 89.66% at 1 × 10^9 conidia/ml. The LC_{50} values for the G-A and G-B strains were calculated to be between 0.2 × 10^6 (0.03–1.6) and 0.6 × 10^6 (0.07–6.1) conidia/ml. Comparable bio efficacy has been reported in other studies evaluating *B. bassiana* isolates against various insect pests, confirming their potential as effective biological control agents.

Sevim et al. (2013) indicated that entomopathogenic fungal isolates tested, the *B. bassiana* isolate KTU-24, originating from *Thaumetopoea pityocampa* (Den. & Schiff., 1775) (Lepidoptera: Thaumetopoeidae), exhibited the highest mortality rate, causing 86% mortality in both adult and nymph stages of *Corythucha ciliata* (Say, 1832) (Hemiptera: Tingidae) within 14 days at a concentration of 1 × 10^7 conidia/ml. The same isolate reached 100% mortality at a higher concentration of 1 × 10^8 conidia/ml.

Utilizing *B. bassiana* as a biopesticide provides an eco-friendly alternative to chemical pesticides, decreasing ecological damage and supporting sustainable agriculture (El-Maraghy et al., 2023; Eko et al., 2024). While *B. bassiana* shows promise, its interaction with the gut microbiota can influence its virulence, suggesting a complex relationship that warrants further investigation (Peng et al., 2023).

Eighteen natives *B. bassiana* strains were isolated from *O. japonica* cadavers. Phylogenetic analysis using ITS confirmed their identification and classified them alongside with reference *B. bassiana* isolates from GenBank. The presence of *B. bassiana* in the detected natural infections and the efficacy trials conducted in the laboratory revealed that *B. bassiana* has insecticidal effects on *O. japonica*. These results may have contributed to the decrease in the population of this pest in the Eastern Black Sea Region in recent years.

This study was conducted under laboratory conditions using both nymphs and adults of the target pest. Future research should extend to greenhouse and field trials to further validate these findings. Additionally, the most effective isolate identified in this study could be developed as a mycoinsecticide, potentially contributing to the biological control strategy against the pest.

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